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(54) Title: RECOMBINANT TRANSFERRINS, TRANSFERRIN HALF-MOLECULES AND MUTANTS THEREOF

(57) Abstract

Recombinant transferrin, transferrin half-molecules and mutant transferrins having altered metal-binding or other properties are described. The recombinant transferrin molecules are expressed in functional form by stable eukaryotic cell lines such as baby hamster kidney cells transformed with an expression vector encoding the recombinant molecule. The recombinant transferrins can be used in metal chelation therapy to bind and clear excess toxic metals in patients suffering from metal overloads.

RECOMBINANT TRANSFERRINS, TRANSFERRIN HALF-MOLECULES AND MUTANTS THEREOF

Background of the Invention

The iron-binding pseudoglobulins collectively
05 called transferrins or siderophilins comprise a class
of proteins with strikingly similar features. X-ray
crystallographic analyses of human lactoferrin
(Anderson, B.F. et al. (1987) Proc. Natl. Acad. Sci.
USA 84:1769-1773) and rabbit serum transferrin
10 (Bailey, S. et al. (1988) Biochemistry 27:5804-5812)
reveal that these proteins consist of two similar
lobes connected by a short bridging peptide and that
each lobe contains two domains defining a deep cleft
containing the binding site for a metal ion and a
15 synergistic anion.

Chicken ovotransferrin gene has been expressed in
transgenic mice (McKnight, G.S. et al. (1983) Cell
(Cambridge, MA) 34:335-341) and a fusion protein of
part of rat transferrin with galactosidase has been

expressed in E. coli (Aldred, A. et al. (1984)
Biochem. Biophys. Res. Commun. 122:960-965). Except
for this fusion protein, attempts to express
transferrin or portions of the molecule in prokaryotic
05 systems have been unsuccessful (Aldred, A. et al.
(1984) Biochem. Biophys. Res. Commun. 122:960-965).
The highly convoluted structure of the protein and
large number of disulfide bridges in the molecule are
probably the major impediments to expression in
10 bacterial hosts. Attempts to mimic partially the
natural protein folding environment by targeting the
protein for bacterial membrane transport via an
attached alkaline phosphatase signal sequence have
been unsuccessful.

15 Summary of the Invention
        This invention pertains to recombinant
transferrin, to recombinant transferrin half-
molecules comprising at least the metal-binding
domains of a single lobe (amino-terminal or
20 carboxy-terminal) of transferrin and to stable cell
culture system for expression of the transferrin. The
recombinant transferrin can be expressed in stable,
transformed eukaryotic cells, such as baby hamster
kidney cells, to yield essentially homogeneous
25 (monodisperse) preparations of the full or
half-molecule forms. The invention also pertains to
mutant transferrins and transferrin half-molecules
which have metal-binding or other properties which are
different from the natural (wild-type) form of the
30 transferrin. These include mutant transferrins and
transferrin half-molecules which bind iron or other
metals more or less avidly than natural transferrin.

Transferrin half-molecules can be used in metal chelation therapy to treat individuals affected with abnormalities of metal regulation or with metal poisoning. For example, transferrin half-molecules,
05 especially mutant forms which bind iron with a higher avidity than natural transferrin, can be administered to iron-overloaded individuals, e.g., thalassemics, in order to clear excess toxic iron from their bodies. In addition, half-molecules, or mutants thereof having
10 altered metal ion selectivities, could be used to clear other toxic metals, e.g., lead, mercury, cadmium, copper and zinc from the body.

Description of the Figures
Figure 1 shows construction of the hTF/2N
15 expression vector in pNUT. A 2.3-kb cDNA encoding human serum transferrin was isolated from a human liver cDNA library and a 1.5-kb PstI/HaI fragment containing the complete amino-terminal domain coding sequence was cloned into M13mp18. Double
20 translational stop codons and a HindIII recognition sequence were introduced by site-directed mutagenesis, allowing the isolation of a BamHI/HindIII fragment which, when joined to a BamHI/HpaII fragment, encodes the amino-terminal domain and signal sequence. This
25 fragment was cloned into the eukaryotic expression vector pNUT, giving the vector pNUT-hTF/N2. In this plasmid, the transferrin cDNA is under the control of the metallothionein promoter (MT-1 pro) and the human growth hormone transcription termination signals
30 (hGH3'); pNUT also contains the SV40 early promoter (SV40) driving expression of a resistant DHFR cDNA (DHFR cDNA) using transcription termination signals from human hepatitis B virus (HBV).

Figure 2 shows a Western blot of immuno-
precipitates from various baby hamster kidney cell
lines. Samples of cell lysates (a) and medium (b)
from Zn-induced cell cultures were precipitated with
05 anti-hTF antiserum. Samples of the resuspended
pellets were analyzed by NaDodSo$_4$-PAGE, transferred to
nitrocellulose and developed with anti-hTF antiserum
followed by alkaline phosphatase conjugated anti-IgG.
The hGH-pNUT and hTF/N2-pNUT cell lines were selected
10 in 500 μ M MTX and all cell culture was performed in
DMEM/10% fetal calf serum. Lane 1, BHK cells; lane 2,
hGH-pNUT transfected BHK cells; lane 3, hTF/N2-pNUT
transfected BHK cells. The positions of molecular
weight markers (x 10^{-3}) are indicated to the right of
15 the blot, the position of the additional protein band
of M_r 37,000 is also indicated (<37) to the right of
the blot.

    Figure 3 shows the isolation and PAGE analysis of
hTF/2N. (Panel A) FPLC isolations on a column of
20 Polyanion SI of recombinant hTF/2N (upper trace) and
proteolytically derived hTF/2N (lower trace). (Panel
B) NaDodSO$_4$-PAGE (5-12% gradient of acrylamide) of
molecular weight standards (lane Mr) and 3 μg of each
of peaks a-d from panel A. (Panel C) Urea-PAGE under
25 nonreducing conditions of the FPLC peaks a-d
(recombinant hTF/2N species) and peaks e-h
(proteolytically derived hTF/2N species) from panel
A. The positions of the apo-protein (apo) and
iron-bound protein (Fe) are indicated. The conditions
30 used for FPLC are given under Materials and Methods.
FPLC fractions were pooled as follows; peak a
(fractions 23-27), peak b (28-31), peak c (32-38),
peak d (39-45), peak e (28-31), peak f (32-36), peak g
(38-44), and peak h (46-51).

Figure 4 shows titration of the major form recombinant hTF/2N with 10 mM Fe(III)(NTA)$_2$. The amount of protein was 3.68 A_{280} units in 1.00 mL of 10 mM NaHCO$_3$. Visible spectra were run 5-10 minutes

05 after each addition of iron to the magnetically stirred cuvette.

Figure 5 shows proton magnetic resonance spectra of recombinant hTF/2N. (a) Fourier transform spectrum with a line broadening of 2 Hz. (b) Convolution

10 difference spectrum with a line broadening of 4 Hz and DC = 4.0, NS = 68 500. The protein sample was 8 mg in 0.1 mL of 0.1 M KCl in ^{2}H$_2$O.

Figure 6 shows the ^{19}F nuclear magnetic resonance spectrum of m-F-Tyr recombinant hTF/2N. The figure

15 shows a Fourier transformation with a line broadening of 10 Hz, NS = 30,000. The protein sample was 6 mg in 0.1 mL of 0.1 M KCl in ^{2}H$_2$O; the reference was 0.1 M trifluoroacetic acid in ^{2}H$_2$O.

Figure 7 shows two separate oligonucleotides used

20 as PCR primers to create the hTF/2C coding sequence. An EcoRI restriction fragment including coding sequence for the entire carboxy lobe was used as a template for 25 rounds of PCR amplification. Oligonucleotide 1 includes a SmaI recognition site and

25 the natural hTF signal sequence at its 5' end and matches the coding sequence for amino acids 334 -341 of hTF at its 3' end. Oligonucleotide 2 matches sequence in the 3' untranslated region of the hTF cDNA and introduces a second SmaI recognition sequence at

30 this site.

Detailed Description of the Invention

This invention provides for the production of
recombinant transferrin, recombinant transferrin
half-molecules and mutant forms of full-length
05 transferrin and transferrin half-molecules which have
altered properties, such as improved metal-binding
capability, compared to the natural transferrin
molecules. Recombinant transferrins can be produced
in large quantities and in substantially homogeneous
10 (monodisperse) form. For example, recombinant
half-molecules of human serum transferrin can be
produced as an essentially homogeneous preparation
substantially free of other human serum proteins. In
contrast, half-molecules prepared by proteolysis of
15 the holo-protein are difficult to purify and, in fact,
the carboxy-terminal half of human transferrin cannot
be satisfactorily prepared by proteolytic means.
Recombinant techniques also allow the application of
mutagenesis to design and produce new forms of
20 transferrin.

In general, a recombinant transferrin of this
invention is produced by transfecting a suitable host
cell with a nucleic acid construct encoding the
transferrin, culturing the transfected host cell under
25 conditions appropriate for expression and recovering
the recombinant transferrin expressed by the cell.
The amino acid sequences for five transferrins have
been reported (Jeltsch, J.-M. and Chambon, P. (1982)
Eur. J. Biochem. 122:291-295; MacGillivray, R.T.A. et
30 al. (1983) J. Biol. Chem. 258:3543-3553;
Metz-Boutigue, M.-H. et al. (1984) Eur. J. Biochem.
145:659-676; Rose, T.M. et al. (1986) Proc. Natl.
Acad. Sci. USA 83:1261-1265; Baldwin, G.S. and

Weinstock, J. (1988) Nucleic Acids Res.
16:8720-8730). The DNA sequence for human serum
transferrin has been determined (Yang, F. et al.
(1984) Proc. Natl. Acad. Sci. USA 81:2752-2756).

05   Full-length DNA for production of recombinant
transferrins or truncated DNA encoding either the
amino-terminal or carboxy terminal lobe of transferrin
or a portion thereof can be obtained from available
sources or can be synthesized according to the known

10   sequences by standard procedures. In order to provide
for secretion of the recombinant transferrin into cell
culture medium, DNA encoding a transferrin signal
sequence (or other signal sequence suitable for the
expression system) is positioned upstream of the

15   transferrin encoding DNA.

Mutant forms of transferrin and transferrin
half-molecules can be produced by standard techniques
of site-directed mutagenesis. See Taylor et al.
(1985) Nucleic Acids Res. 13:8749-8764; Zoller, M.J.

20   and Smith, M. (1983) Meth. Enzymol 100:458-500. In
particular, mutagenesis can be used to produce mutant
transferrins which have metal binding properties that
are different from natural transferrin. For example,
mutants capable of binding iron more avidly than

25   natural transferrin can be produced. To produce such
mutants metal-binding domains can be mutagenized to
replace one or more amino acids involved in binding
with different amino acids. In human serum
transferrin, the amino acids which are ligands for

30   metal chelation are shown below (the number beside the
amino acid indicates the position of the amino acid
residue in the primary sequence where the first valine
of the mature protein is designated position 1)

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Amino terminal lobe (amino acids 1-337)		Carboxy terminal lobe (amino acids 343-679)	
Aspartic acid	63	Aspartic acid	392
Tyrosine	95	Tyrosine	426
05 Tyrosine	188	Tyrosine	517
Histidine	249	Histidine	584

In other types of transferrin, the numbering is different, the ligands (amino acids) are the same.

Other regions of transferrin control binding and
10 these too can be targeted for mutagenesis. These are
usually positively charged amino acids such as lysine,
histidine or arginine. For example, a mutant
transferrin half-molecule which binds iron more avidly
than natural transferrin can be produced by replacing
15 the lysine residue at position 206 with glutamine
(AAG→CAG).

The transferrin-encoding DNA is cloned into a
eukaryotic expression vector containing appropriate
regulatory elements to direct expression of the DNA.
20 A preferred eukaryotic expression vector is the
plasmid pNUT described by Palmiter, R.D. et al. (1987)
Cell 50:435-443. This plasmid contains the
metallothionein promoter which includes transcription
of the transferrin encoding DNA in the presence of
25 heavy metal and transcription termination signals of
human growth hormone. In addition, pNUT contains
dihydrofolate reductase gene under control of the SV40
early promoter with transcription termination signals
from human hepatitis B virus to allow selection in
30 cell culture. The gene encodes a mutant form of the
enzyme which has a 270-fold lower affinity for the
competitive inhibitor methotrexate. This allows for

the immediate selection of transfected cells in very
high concentrations (0.5 mM) of methotrexate and
abrogates the need for a recipient cell line that is
deficient in dihydrofolate reductase. pNUT also
05 contains pUC18 derived sequences which allows it to be
amplified in E. coli to provide sufficient amounts of
the plasmid for transfection of recipient cells.

The expression vector containing the DNA encoding
the transferrin is incorporated into an appropriate
10 host cell. The preferred host cell is a eukaryotic
cell which can be transformed with the vector to yield
a stable cell line which expresses a functionally
active transferrin construct. A particularly useful
cell is the baby hamster kidney cell. Baby hamster
15 kidney cells can be transfected with a vector carrying
the DNA construct encoding a transferrin (such as the
pNUT plasmid) to provide a stable cell culture system
which expresses and secretes a functionally active
transferrin (full or half-molecule). These cells are
20 well-suited for economical, large scale growth and can
be obtained from readily available sources.

Standard techniques, such as calcium phosphate
coprecipitation or electroporation can be used to
transfect the eukaryotic host cell with the vector.
25 The cell is then cultured under conditions appropriate
to induce expression of the transferrin. For example,
baby hamster kidney cells transfected with the pNUT
vector are stimulated to express the transferrin
construct in the presence of heavy metals. Baby
30 hamster kidney cells are preferably cultured in the
medium Dulbecco's Modified Eagle's medium-Ham's F-12
nutrient mixture with the serum substitute Ultraser G™
(Gibco) at about 1%.

After an appropriate culture period, the expressed and secreted transferrin can be recovered from the culture medium. Standard purification procedures can be employed to yield a substantially homogeneous preparation of the recombinant transferrin. In one embodiment, the transferrin in the culture medium is saturated with iron and then purified by anion exchange chromatography.

The recombinant transferrins of the invention can be used to chelate and clear iron or other toxic metals from the body. The customary approach to iron chelation in vivo has been to assess a wide variety of naturally-occurring siderophores of microbial origin and synthetic iron chelators for their physiological effects, primarily the ability to bind and clear iron from the body. Many such compounds have been studied with varying abilities to clear iron and often with unacceptable side effects (Pitt, C.G. et al. (1979) J. Pharm. Exp. Therap. 208:12-18). As a result, the only iron chelator used for clearing excess iron from humans remains deferoxamine, a cyclic peptide from Streptomyces pilosis.

A preferred transferrin for iron chelation therapy is a mutant transferrin half-molecule which binds iron more avidly than natural transferrin. The use of a mutant half-molecule allows for more efficient chelation and removal of the metal. A particularly preferred mutant half-molecule is K206Q, described in the Exemplification below, which contains a glutamine rather than a lysine at position 206.

A transferrin half-molecule is advantageous because unlike the holo-proteins, it passes through the glomeruli of the kidney and is excreted in the

urine, so that metal is not only chelated but also cleared from the body. Moreover, the single half-molecules do not bind to transferrin receptors on the membrane of tissue cells and therefore do not

05 deliver iron to these tissues. Further, half-molecules of human transferrin would probably be recognized as "self" by the human body and therefore would not elicit an immunological response.

In addition, mutant half-molecules can be

10 designed to have altered metal ion selectivities. The chelators could be used to clear other toxic metals from the body, e.g., lead, mercury, cadmium, copper and zinc.

For chelation therapy, the recombinant

15 transferrin is administered to a patient in amounts sufficient to chelate the metal and reduce circulating levels below toxic levels. Generally, it is administered in a physiologically acceptable vehicle, such as saline, by a parenteral route (typically

20 intravenously).

Recombinant full-length human transferrin can be used in nonserum supplements for cell culture media. Transferrin is required for iron uptake by growing cells. The use of recombinant transferrin avoids the

25 risk of contamination (with, e.g., HIV or hepatitis virus) associated with transferrin purified from human serum.

The invention is illustrated further by the following exemplification:

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EXEMPLIFICATION

I. Production of Recombinant Transferrin Half-
Molecule Comprising the Amino-Terminal Lobe.

MATERIALS

05   T4 DNA ligase, DNA polymerase I (Klenow fragment)
and T4 polynucleotide kinase were purchased from
Pharmacia-PL Biochemicals. Restriction endonucleases
were purchased from Pharmacia-PL Biochemicals and
Bethesda Research Laboratories. Oligodeoxyribo-
10 nucleotides were synthesized on an Applied Biosystems
380A DNA Synthesizer. Nitrocellulose filters were
obtained from Schleicher and Schuell, ^{32}P-labeled
nucleotides from New England Nuclear, goat anti-human
transferrin antiserum from the Sigma Chemical Company,
15 formalin-fixed Staphylococcus aureus cells from
Bethesda Research Laboratories, the Protoblot
immunoscreening detection system from Promega, the
oligonucleotide-directed mutagenesis kit from
Amersham, Dulbecco's modified essential medium and
20 fetal bovine serum from Gibco, and anti-human
transferrin monoclonal antibody HTF-14 was from the
Czechoslovakian Academy of Sciences. All other
reagents were analytical grade or purer.

METHODS

25   Isolation of Human Serum Transferrin (hTF) cDNA.
A human liver cDNA library constructed in the E. coli
expression vector pKT-218 (Prochownik, E.V. et al.
(1983) J. Biol. Chem. 258:8389-8394) provided by Dr.
Stuart Orkin, (Harvard University) was screened using
30 a synthetic oligonucleotide coding for the

amino-terminal eight amino acids of serum hTF as a
hybridization probe. The oligonucleotide corresponded
to nucleotides 88 to 111 of the hTF cDNA sequence
reported by Yang, F. et al. (1984) Proc. Natl. Acad.
05 Sci. USA 81:2752-2756). The oligonucleotide was
end-labeled with T4 polynucleotide kinase and ^{32}P-ATP
(Chaconas, G. and van de Sande, J.H. (1980) Methods
Enzymol. 65:75-85), and used to screen approximately
10^5 colonies. Restriction endonuclease mapping of
10 positive clones and DNA sequence analysis were
performed by using standard procedures with pUC19 and
M13mp19 vectors, respectively (Maniatis, T. et al.
(1982) Molecular Cloning, a Laboratory Manual, Cold
Spring Harbor Laboratory, Cold Spring Harbor, NY;
15 Messing, J. (1983) Methods Enzymol. 101:20-78; Sanger,
F. et al. (1977) Proc. Natl. Acad. Sci. USA
74:5463-5467).
     Expression Vector and Cell Culture. The
eukaryotic expression vector pNUT (Palmiter, R.D. et
20 al. (1987) Cell (Cambridge, MA) 50:435-443) and baby
hamster kidney (BHK) cells were provided by Dr.
Richard D. Palmiter (Howard Hughes Medical Institute,
University of Washington). After synthesis,
oligonucleotides were purified on C_{18} reverse-phase
25 columns (Sep-Pak, Waters Associates; Atkinson, T. and
Smith, M. (1984) Oligonucleotide Synthesis: A
Practical Approach (Gait, M.J., Ed.) pp 35-81, IRL
Press, Oxford). Site-directed mutagenesis was
performed by using the method of Taylor, J.W. et al.
30 (1985) Nucleic Acids Res. 13:8749-8764). Plasmid DNA
was prepared from E. coli JM105 and purified by two
successive centrifugation steps with cesium chloride
density gradients.

BHK cells were grown in Dulbecco's modified
essential medium (DMEM) with 10% fetal bovine serum to
approximately 10^7 cells per 10-cm dish and were
subsequently transfected with 10µg of plasmid by the
05 calcium phosphate co-precipitation technique described
by Searle, P.F. et al. (1985) Mol. Cell. Biol.
5:1480-1489). After 24 hours, the medium was changed
to DMEM containing 100 µM methotrexate (MTX) and
surviving cells were serially selected to 500 µM MTX.
10 In some experiments, cells were selected immediately
with 500 µM MTX. Large scale roller bottle cultures
were initiated by seeding approximately 5×10^7 cells
into each 850 cm^2 roller bottle containing 100 mL of
DMEM-MTX. Cultures were induced at 80% confluency by
15 the addition of $ZnSO_4$ to the medium to a final
concentration of 0.08 mM. The medium was harvested 40
hours later.

    Immune-precipitation and Western Blotting.
Immune-precipitation of cell culture medium and cell
20 lysates was performed by the method of Van Oost, B.A.
et al. (1986) Biochem. Cell Biol. 64:699-705).
Precipitates were analyzed by electrophoresis on 12%
polyacrylamide gels in the presence of $NaDodSO_4$
(Laemmli, U.K. (1970) Nature (London) 227:680-685),
25 followed by blotting onto a nitrocellulose membrane.
The blot was incubated in PBS containing 0.1 mg/ml
gelatin, then treated with goat anti-hTF antiserum
(250-fold dilution in PBS), and finally developed with
an alkaline phosphatase-conjugated, rabbit anti-goat
30 IgG antibody according to the supplier's instructions.

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Amino Acid Substitution. To incorporate
3-fluorotyrosine into the recombinant hTF/2N as a ^{19}F
NMR probe, the culture medium was supplemented with
D,L-m-fluorotyrosine (Sigma Chemical Company) at 16%
05 of the concentration of L-tyrosine in the medium. The
cells grew as well on this medium as on the medium
lacking D,L-m-fluorotyrosine.

Isolation of Recombinant hTF/2N. Harvested
culture medium was made 0.01% in phenylmethylsulfonyl
10 fluoride to inhibit proteases and sufficient
Fe(III)(NTA)$_2$ was added to saturate all transferrin in
the medium. After stirring at room temperature, the
solution was dialyzed for 24 hours versus cold running
tap water, and then for a few hours versus Milli-Q
15 purified water. Concentrated Tris-HCl buffer, pH 8.4
was added to a final concentration of 5 mM, the
preparation was centrifuged to remove any debris, and
was loaded onto a column (2.5 x 80 cm) of
DEAE-Sephacel (Pharmacia) equilibrated with 10 mM
20 Tris-HCl buffer, pH 8.4.

The column was then eluted with a linear gradient
of NaCl (0 to 0.3 M) in the same buffer. Fractions
showing a pink color were analyzed by NaDodSO$_4$-PAGE,
and fractions containing the recombinant protein (Mr
25 37,000) were pooled. Such fractions also contained
bovine transferrin and albumin resulting from the
fetal calf serum in the tissue culture medium. After
concentration of the pooled fractions to 5 mL on an
Amicon PM-10 membrane, the protein was chromatographed
30 on a column (2.5 x 90 cm) of Sephadex G-75 Superfine
(Pharmacia-PL Biochemicals) equilibrated with 100 mM
ammonium bicarbonate.

Sometimes, a second chromatographic step through
this column was necessary to resolve completely the
hTF/2N from the bovine proteins. At this stage, the
A_{465}/A_{410} was usually < 1.0, indicating the presence
05 of a contaminating heme-protein (possibly hemopexin).
The hTF/2N was finally purified to homogeneity by FPLC
on a column (1 x 10 cm) of Polyanion SI (Pharmacia)
using a linear gradient of NaCl (O to 0.3 M) in 50 mM
Tris-HCl, pH 8.0 over a period of an hour at a flow
10 rate of 1ml/min. Fractions of 1 mL were collected.
Two to four protein bands emerged from the column,
depending on the iron-binding status of the protein.

NaDodSO$_4$-PAGE was performed with 5% to 12%
gradient gels and urea-PAGE was performed according to
15 a modification (Brown-Mason, A. and Woodworth, R.C.
(1984) J. Biol. Chem. 259:1866-1873) of the Makey,
D.G. and Seal, U.S. (1976) Biochim. Biophys. Acta
453:250-256 procedure. Electrofocusing was performed
on a 0% to 50% sucrose gradient in a 110 mL glass
20 column (LKB) with 0.8% Pharmalyte, pH 5 to 8
(Pharmacia). The column was prefocused overnight to a
final current of 2 mA at 1000 V.

The protein sample in 0.2 mL was diluted with 5
mL of solution withdrawn from the middle of the
25 gradient. The sample was then reinjected into the
isodense region of the column and focusing was
continued for 24 hours. The gradient was collected
from the bottom of the column in 1.5 mL fractions.
Individual fractions were analyzed for A_{280} and for
30 pH. Fractions with maximum A_{280} were selected as
representing the pIs of the apo- and iron-saturated
proteins.

Iron was readily removed from the iron-protein by
incubation in a buffer containing 1 mM NTA, 1 mM EDTA,
0.5 M sodium acetate, pH 4.9. The apo-protein was
concentrated to a minimum volume on a Centricon 10
05 (Amicon), then diluted and reconcentrated twice with
water and twice with 0.1 N KCl. The apo-protein had a
tendency to precipitate in pure water, but redissolved
readily in 0.1 M KCl. The apo-protein was made 10 mM
in NaHCO$_3$ and titrated with a suitable concentration
10 of Fe(NTA)$_2$ while monitoring the absorbance at 465 nm.

Quantitative Immunoassay of Recombinant hTF/2N.
A competitive solid state immunoassay was used to
assess the concentration of recombinant hTF/2N in the
culture fluid and at various stages of the
15 purification (Foster, W.B. et al. (1982) Thromb. Res.
28:649-661). Proteolytically-derived Fe-hTF/2N
(Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem.
255:708-713) was radioiodinated (Fraker, P.J. and
Speck, J.C., Jr. (1978) Biochem. Biophys. Res. Commun.
20 80:849-857) with Iodogen (Pierce Chemical Company) and
used as the standard. The monoclonal anti-hTF
antibody HTF-14 was used as the probe (Bartek, J. et
al. (1984) Folia Biol. (Prague) 30:137-140). This
antibody recognizes only the amino-terminal lobe of
25 hTF (Mason, A.B. et al. (1988) Br. J. Haematol.
68:392-393) and does not recognize bovine transferrin
(Penhallow, R.C. et al. (1986) J. Cell. Physiol.
128:251-260).

Amino-terminal Sequence Analysis. The
30 amino-terminal sequences of both the minor and
major-forms of recombinant hTF/2N were determined on
an Applied Biosystems 470A Protein Sequencer in the
Given Analytical Facility at the University of Vermont.

Periodic Acid-Schiff Stain. The presence of oligosaccharides in the recombinant hTF/2N was determined by staining the protein with periodic acid-Schiff reagent (Fairbanks, G. et al. (1971)

05 Biochemistry 10:2606-2617).

Nuclear Magnetic Resonance Spectroscopy. Proton and fluorine NMR spectra were obtained on the 5.872 Tesla Bruker WM NMR spectrometer in the Camille and Henry Dreyfus NMR Laboratory, Department of Chemistry,

10 University of Vermont, operating in the Fourier transform mode with quadrature detection. An ^{19}F probe was provided by Dr. Christopher W. Allen of that department. For proton spectra, spectrometer settings were as described previously (Valcour, A.A. and

15 Woodworth, R.C. (1987) Biochemistry 26:3120-3125). For ^{19}F spectra, the sweep width was 30,000 Hz, the acquisition time was 0.279 seconds, a receiver delay of 2.0 seconds intervened between acquisition and pulse of 15.0 1s (90°) and the sample was at 303°K.

20 ^{19}F chemical shifts are relative to 0.1M trifluoroacetic acid in $^{2}H_2O$. Protein samples were 6 to 8 mg in 0.1 mL of 99.8 atom% $^{2}H_2O$, and spectra were run on these samples in 0.1 mL capsules inserted into standard 5 mm NMR tubes containing $^{2}H_2O$. Free

25 induction decays of ^{19}F spectra were subjected to a line-broadening of 10 Hz prior to Fourier transformation.

RESULTS

Isolation of Human TF cDNA. Approximately

30 100,000 colonies of a human liver cDNA library (Prochownik, E.V. et al. (1983) J. Biol. Chem. 258:8389-8394) were screened by using a 24 base

oligonucleotide to the 5' sequence of the human TF
cDNA as a hybridization probe. A single positive
colony was obtained. Extensive restriction enzyme
mapping of the plasmid isolated from this clone agreed
05 completely with the patterns predicted from the human
TF cDNA isolated from the same library by Yang, F. et
al. (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756.
DNA sequence analysis of the 5'- and 3'-termini of
this clone confirmed that it was identical to the
10 full-length clone isolated by Yang et al. All
subsequent sequence analysis performed during the
mutagenesis and subcloning of this cDNA conformed
exactly to the sequence reported previously.

Vector Construction and Expression. Two
15 translational stop codons and a unique HindIII
recognition site were introduced into the linker
region between the amino- and carboxy-terminal domains
of the hTF cDNA sequence by oligonucleotide-directed
mutagenesis. The predicted translation sequence from
20 this construct ends at Asp-337, according to the serum
hTF numbering sequence (MacGillivray, R.T.A. et al.
(1983) J. Biol. Chem. 258:3543-3553).

The expression vector pNUT (Palmiter, R.D. et al.
(1987) Cell (Cambridge, MA) 50:435-443) contains a
25 mouse metallothionein-1/human growth hormone gene
fusion that has been shown to direct high levels of
human growth hormone in transgenic mice (Palmiter,
R.D. et al. (1983) Science (Washington, D.C.)
222:809-814). Important functional features of this
30 vector include a mouse metallothionein-1 promoter to
induce cDNA transcription in the presence of heavy
metals, pUC18 sequences to allow replication and
selection in E. coli, and a dihydrofolate reductase

(DHFR) cDNA driven by the SV40 early promoter to allow selection in cell culture. The DHFR cDNA encodes a mutant form of the enzyme which has a 270-fold lower affinity for the competitive inhibitor methotrexate

05 (MM) (Simonsen, C.C. and Levinson, A.D. (1983) Proc. Natl. Acad. Sci. USA 80:2495-2499). This allows for the immediate selection of transfected cells in very high concentrations (0.5 mM) of MTX and abrogates the need for a recipient cell line that is deficient in

10 DHFR.

　　To construct the expression vector pNUT-hTF/2N, the BamHI-HindIII fragment from the bacterial expression vector was isolated (Figure 1). A HpaII-BamHI fragment from the original transferrin

15 cDNA clone was also isolated (Figure 1). These two fragments were then ligated into M13mpl8 replicative form DNA that had been cut with AccI and HindIII. Replicative form DNA from the resulting M13 phage was isolated, the insert released by cleavage with XbaI

20 and HindIII, and the ends made blunt ended. These steps ensured that the fragment included the translational stop signals, retained the natural signal sequence for the protein, and was free of the dG/dC tail found in the original vector (Figure 1).

25 This fragment was inserted into SmaI-cut pNUT, thus replacing the human growth hormone gene with a hTF/2N encoding cDNA, but leaving the transcriptional termination signal from the growth hormone gene intact. This plasmid was transfected into BHK cells

30 and the resulting transformants were selected in the presence of MTX.

To analyze the mRNA transcripts produced by the
transfected BHK cells, total RNA was electrophoresed
on an agarose gel in the presence of formaldehyde
(Maniatis, T. et al. (1982) Molecular Cloning, a
05 Laboratory Manual, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY). After transfer to nitrocellulose,
the blot was analyzed by using an oligonucleotide to
the 3' untranslated region of the hGH gene as a
hybridization probe. An inducible mRNA of
10 approximately 1.4 kb was detected in the transfected
cell line but not in mock-infected BHK cells (data not
shown). This agreed with the predicted size of the
hTF/2N mRNA, including the expected hGH 3'
untranslated sequence and poly (A) tail.
15   To analyze the polypeptides produced by the
transformed BHK cells, Western blot analysis was
performed both on cell lysates and the medium of
various cell lines (Figure 2). Samples of BHK cells,
BHK cells containing the hGH-pNUT plasmid, and BHK
20 cells containing the hTF/2N-pNUT plasmid were grown in
DMEM (BHK cells) or DMEM-MTX (BHK cells containing
pNUT vectors). When the cells were reaching
confluence, samples of medium were taken and cell
lysates were prepared. These samples were incubated
25 successively with goat anti-hTF antiserum and
formalin-fixed S. aureus cells (Van Oost, B.A. et al.
(1986) Biochem. Cell Biol. 64:699-705).
Bound proteins were eluted by incubation with
NaDodSO$_4$, electrophoresed on a polyacrylamide gel, and
30 transferred to a nitrocellulose membrane. The
membrane was then incubated with goat anti-hTF
antiserum and rabbit anti-goat immunoglobulin
conjugated to alkaline phosphatase. When cell lysates

or medium from BHK cells (Figure 2, lanes 1a and 1b)
or BHK cells with hGH-pNUT plasmid (Figure 2, lanes 2a
and 2b) were analyzed, only the expected goat
immunoglobulin bands (Mr 25,000 and 50,000) from the
05 original goat anti-hTF antibodies and a small amount
of cross-reacting material were observed. However, an
additional band of Mr 37,000 was observed in cell
lysates (Figure 2, lane 3a) or medium (Figure 2, lane
3b) of the BHK cells containing the hTF/2N-pNUT
10 plasmid. The molecular weight of this polypeptide
chain is in excellent agreement with the molecular
weight of the hTF/2N molecule (37,833) calculated from
the amino acid sequence.

          The homogeneity of the hTF/2N product indicates
15 the successful removal of signal sequence as cell
lysate and secreted samples comigrate on SDS-PAGE.
The anti-serum appears to be highly specific for human
TF species, since little bovine TF is apparent in the
precipitates.

20   In large scale cultures of the hTF/2N cell line
grown in roller-bottles, the concentration of hTF/2N
in the medium was approximately 10-15 µg/ml as
detected by radioimmunoassay.

          Isolation and Characterization of Recombinant
25 hTF/2N. Recombinant hTF/2N was purified by a
three-step procedure that led routinely to an 80%
yield of the major form of the protein, based on
radioimmunoassay. The final purification on Polyanion
SI led to quantitative resolution of the apo- and
30 iron-saturated forms of both the minor (<5%) and major
constituents of the protein (Figure 3, panel A), as
corroborated by urea-PAGE (Figure 3, panel C). Note
that on urea-PAGE the slowest moving bands are

apo-hTF/2N and the faster moving bands are Fe-hTF/2N.
SDS-PAGE gels (Figure 3, panel B) showed the major and
minor forms of recombinant hTF/2N to be monodisperse,
of equal molecular weight and the major component to
05 be free of carbohydrate by PAS stain (data not
shown).

     In general these preparations appear to have
better monodispersity than proteolytically derived
hTF/2N (Lineback-Zins, J. and Brew, K. (1980) J. Biol.
10 Chem. 255:708-713) (Figure 3). For example, the
chromatographic peaks are more regular for the former,
and the number of bands on urea-PAGE is greater for
the latter. Spectral ratios for the iron-saturated
recombinant protein are typically A_{280}/A_{465} =21 and
15 A_{465}/A_{410} = 138, which compare favorably with values
for pure diferric transferrin isolated from human
plasma. Titration of 3.68 A_{280} units of the
apo-protein with $Fe(NTA)_2$ yields a slope corresponding
to an $E_{465}mM$ = 2.1 and gives for the apo-protein
20 $E_{280}mM$ = 38.8 (Figure 4), both reasonable values for a
half-transferrin molecule (Lineback-Zins, J. and Brew,
K. (1980) J. Biol. Chem. 255:708-713; Zak, O. et al.
(1983) Biochim. Biophys. Acta 742:490-495). The pI's
for the apo- and Fe-hTF/2N were 6.5 and 5.4,
25 respectively.

     Amino-terminal sequence analysis of both the
minor and major forms of recombinant hTF/2N gave
results identical to those found (MacGillivray, R.T.A.
et al. (1983) J. Biol. Chem. 258:3543-3553) for
30 holo-hTF from serum (Table 1).

The proton NMR spectrum of the recombinant protein (Figure 5) is very similar to that for the proteolytically-derived hTF/2N (Valcour, A.A. and Woodworth, R.C. (1987) Biochemistry 26:3120-3125), but the resonance lines are sharper for the recombinant protein. The ^{19}F NMR spectrum of the protein derived from a cell culture grown on medium supplemented with m-F-tyrosine (Figure 6) shows four well-resolved resonances, two possibly having an unresolved shoulder.

Table 1

Amino-Terminal Sequence of Human Transferrin and of the Recombinant Human Transferrin Amino-Terminal Half-Molecule

Protein	Amino Acid Sequence	Reference
human serum transferrin	V-P-D-K-T-V-R-W-C-A-V-S-	MacGillivray et al. (1983)
recombinant hTF/2N (major)	V-P-D-K-T-V-R-W-X-A-V-S-	this report
recombinant hTF/2N (minor)	V-P-D-K-T-V-	this report

[a]The recombinant hTF/2N sequences were determined on an Applied Biosystems 470A protein sequencer. Approximately 200 pmol of each sample was analyzed. [b]Twelve sequencer cycles were analyzed. [c]No residue was identified at cycle 9; however, cysteine residues were not modified prior to the analysis. [d]Six sequencer cycles were analyzed.

By using recombinant DNA technology, a hTF/2N
molecule is produced that functions identically with
the proteolytically derived species as judged by
several independent criteria. This represents the
05 first reported expression in a stable cell culture
system of a functionally active form of this important
iron transport protein.

The pNUT based hTF/2N construction described here
produces high levels of recombinant protein without
10 the need for a DHFR-deficient cell line or tedious
resistance amplification procedures. BHK cells are
well-suited for economical, large scale growth and we
are currently examining their growth characteristics
on micro-carrier supports in bioreactor vessels. By
15 using either roller bottles or a fermentor with a
capacity of several liters, we can easily produce
sufficient recombinant protein even for techniques
such as NMR that traditionally have required a high
concentration of protein.

20   The minor form of recombinant hTF/2N isolated on
Polyanion SI migrates more slowly than the major form
on urea-PAGE (Figure 3, panel C), but at the same rate
on SDS-PAGE (Figure 3, panel B). Thus, the apparent
molecular weights are the same but the relative
25 degrees of unfolding in 6 M urea differ. Note that
the proteolytically-derived apo-hTF/2N shows even
faster migrating species in 6 M urea (Figure 3, panel
C, fractions g and h).

Contamination of apo-hTF/2N with Fe-hTF/2N and
30 vice versa on these gels arises from the method of
pooling FPLC fractions, from some loss of bound iron
on the urea gel and from binding of contaminating iron
during workup of the FPLC samples. Identical

N-terminal sequences (Table 1) show that the signal
peptide has been removed from both minor and major
forms of the recombinant protein. As in hTF/2N from
human serum (Lineback-Zins, J. and Brew, K. (1980) J.
05 Biol. Chem. 255:708-713), the recombinant hTF/2N is
non-glycosylated. The cause of the difference between
major and minor forms of hTF/2N is unknown at
present. The minor form has never represented more
than 5% of the total recombinant protein and is
10 usually less than 1%. Thus, the goal of isolating a
monodisperse recombinant hTF/2N (the major form) has
been achieved.

The iron binding behavior, pIs, migration on
NaDodSO$_4$-PAGE and urea-PAGE and proton NMR spectra of
15 the recombinant hTF/2N match reasonably well those of
the hTF/2N derived from amino terminal monoferric hTF
by proteolysis with thermolysin (Lineback-Zins, J. and
Brew, K. (1980) J. Biol. Chem. 255:708-713; Valcour,
A.A. and Woodworth, R.C. (1987) Biochemistry
20 26:3120-3125), except as noted above. The major form
of the recombinant protein shows a higher degree of
monodispersity (Figure 3) and its proton NMR spectrum
shows sharper resonance lines than does the
proteolytically derived hTF/2N. There has been
25 insufficient minor form for analysis by NMR.

Previous studies of the incorporation of
m-fluorotyrosine into alkaline phosphatase from E.
coli have established the efficacy of ^{19}F NMR for
specifically probing the tyrosyl residues in a protein
30 (Sykes, B.D. et al. (1974) Proc. Natl. Acad. Sci. USA
71:469-473; Hull, W.E. and Sykes, B.D. (1974)
Biochemistry 13:3431-3437). Incorporation of
m-F-tyrosine into the recombinant hTF/2N proves that

selective amino acid substitution is possible in this
cell culture system and gives us access to a specific
NMR probe of tyrosyl side chains. This preparation
behaves in all respects like the non-modified protein
05 as described above for the non-substituted
recombinant. When we have optimized the cell culture
conditions to achieve higher levels of incorporation,
changes in the ^{19}F NMR spectrum on addition of
paramagnetic and diamagnetic metals and on changes in
10 pH will be useful in studying the tyrosyl residues
specifically involved in metal binding. Incorporation
of selectively deuterated aromatic amino acids will
allow us to dissect the aromatic region of the proton
NMR spectrum of the protein in similar fashion to the
15 studies on lysozyme from Japanese quail (Brown-Mason,
A. et al. (1981) J. Biol. Chem. 256:1506-1509).

II. Production of Recombinant Transferrin Half-
Molecule Comprising Carboxy Terminal Lobe.

An EcoRI restriction fragment including the
20 coding sequence for the carboxy lobe of hTF was
isolated from the full length hTF cDNA and then used
as a template for PCR-directed mutagenesis (Figure
2). Two oligonucleotides were synthesized to be used
as PCR primers. Oligo 1 encodes a SmaI recognition
25 site, followed by sequence encoding the natural signal
sequence of hTF, followed by sequence matching the
coding sequence for amino acids 334-341. The second
oligonucleotide matches the complement of the 3'
nontranslated region of the hTF cDNA and introduce a
30 SmaI recognition sequence 3' to the normal translation
termination site (nucleotides 2125-2127 using the

numbering system of Yang, F. et al. (1984) Proc. Natl.
Acad. Sci. USA 81:2752-2756). Twenty-five rounds of
PCR amplification using Taq polymerase (Perkin Elmer)
resulted in the desired DNA fragment which splices the
05 natural signal sequence of hTF to the C lobe coding
sequence. This fragment was then digested with SmaI
and ligated with the large SmaI fragment of pNUT as
for the hTF/2N expression studies.

III. Production of Recombinant Full Length Transferrin.

10   The coding sequence for human serum transferrin
was assembled from restriction enzyme digestion
fragments derived from the full-length cDNA clone
isolated from a human liver library described above.
Since the parental plasmid (pKT-218) of the original
15 clone had a limited number of unique restriction
enzyme recognition sites, a series of cloning steps
was required to introduce the coding sequence into a
convenient vector. This process was initiated by
cloning a HpaII/BamHI fragment from the 5' end of the
20 cDNA into the vector pUC 18 (Messing, J. (1983) Meth.
Enzymol. 101:20-28). The resulting plasmid was
digested with BamHI and HindIII and a BamHI/HindIII
fragment from the human transferrin cDNA was cloned
adjacent to the initial fragment. The resulting
25 plasmid was then digested with HindIII and PstI and a
final HindIII/PstI fragment from the 3' end of the
transferrin cDNA was cloned to complete the assembly
of the full-length coding sequence. Digestion of the
resulting plasmid with SacI and SphI released the

full-length coding sequence as a single restriction
fragment which was subsequently made blunt using T4
DNA polymerase and dNTPs and then cloned into the
large SmaI fragment of pNUT (Palmiter et al. (1987)
05 Cell 50:435-443) as described for the N-and C-terminal
transferrin half-molecule coding sequences.

     Plasmid DNA was prepared from E. coli JM105 and
purified by two successive centrifugation steps with
cesium chloride gradients. Baby hamster kidney (BHK)
10 cells were grown in Dulbecco's Modified Eagles'
medium-Ham's F-12 nutrient mixture (DMEM-F-12) (Gibco;
Sigma) with 10% fetal bovine serum to approximately
10^7 cells per 100 mm dish and were subsequently
transfected with 10 µl of plasmid by the calcium
15 phosphate coprecipitation technique described by
Searle et al. (1985) Mol. Cell Biol. 5:1480-1489.
After 24 hours the medium was changed to DMEM-F-12
containing 500 µM methotrexate to select the plasmid
containing cells. Once selected, the cells were
20 serially passaged at approximately 80% confluency with
phosphate buffered saline containing EDTA (0.2 gm/l)
to five 100-mm dishes, then to five T-175 flasks and
finally to five expanded surface roller bottles (200
ml each). At the T-175 passage, a serum substitute,
25 Ultraser G (Gibco), at a level of 1% was used in place
of fetal calf serum in DMEM-F-12 lacking phenol red.

     It was found that once production levels were
high (approximately 100 µg/ml of medium), medium
without Ultraser G could sustain production of
30 recombinant protein for at least two passages. This
greatly simplified the isolation of the expressed
full-length recombinant human serum transferrin. To
isolate the recombinant protein, harvested culture

medium is made 0.01% with respect to phenylmethanesulfonyl fluoride and sodium azide to inhibit proteases and bacterial growth respectively. Sufficient Fe^{3+} (nitrilotriacetic acid)$_2$ is added to
05 saturate the transferrin present. The medium is reduced in volume to <10 ml and the transferrin is purified by passage over an anion exchange column (Polyanion SI, 1 x 10 cm) as described for the recombinant amino terminal human transferrin
10 half-molecule. See above.

The isolated recombinant full-length human serum transferrin displays some heterogeneity on this column attributed to variation in the glycosylation pattern. The protein is monodisperse on NaDod
15 SO$_4$-polyacrylamide gel electrophoresis and has a spectrum and spectral ratios which are comparable to purified human serum transferrin.

IV. Production of Mutant Transferrins.

Substitution mutants are designated using the
20 conventional single letter amino acid symbol of the wild type (native) residue, followed by the positional number of the replacement in the primary sequence, (where valine of the mature protein is designated position 1) followed by the symbol for the replacement
25 residue. For example, a mutant in which aspartic acid residue at position 63 is replaced by a serine residue would be designated D63S.

The production of hTF/2N mutants was accomplished by two techniques. A D81S substitution was prepared
30 using the method of Nelson, R.M. and Long, G.L. (1989) Analyt. Biochem. 180:147-151. Briefly, a HpaII/BamHI

fragment from the 5' end of the hTF/2N coding sequence
was subcloned into pUC18 and then used as a template
for a two step PCR-based mutagenesis procedure. The
resulting DNA fragment was then recloned into M13mp18
05 and the sequence of the mutant construction was
confirmed by dideoxy sequence analysis. The fragment
was then released from the double stranded form of the
sequencing vector by digestion with XbaI and BamHI and
then ligated to a BamHI/HindIII fragment from the
10 original hTF/2N construction to produce a full length
D81S-hTF/2N coding sequence, the fidelity of this
splicing was confirmed by restriction digestion
analysis and was subsequently cloned into pNUT as
before.

15   The substitution mutants G65R, D63C, K206Q and
H207E were produced by subcloning the entire hTF/2N
coding sequence into M13mp18, which was then used as a
template for oligonucleotide-directed mutagenesis
(Zoller, M.J. and Smith, M. (1983) Meth. Enzymol.
20 100:458-500) using the dut⁻, ung⁻ selection procedure
(Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA
82:488-492). Following mutagenesis, the entire coding
sequence for the mutant sequences was confirmed by
dideoxy sequence analysis using sequencing primers
25 targeted along the length of the coding sequence at
250 bp intervals. The desired coding sequences were
then released by restriction digestion, made blunt and
inserted into pNUT as before.

        pNUT plasmids have been constructed containing
30 the cDNA a) for full-length human serum transferrin
(hTF) and b) for various site-directed mutants of the
amino-terminal half-molecule (hTF/2N). These mutants
include 1) D63S patterned on the naturally occurring

mutation found in the C-terminal half of human
melanoferrin, b) G65R patterned on the naturally
occurring mutant found in the C-terminal half of hTF
from a patient in England, c) K206Q based on the wild
05 type mutation in the C-terminal half of ovotransferrin
(oTF) from hen's egg white, d) H207E based on the wild
type mutation in human lactoferrin (hLTF) and e) D63C
as an attempt to change the metal selectivity of the
iron binding site. All of these constructions have
10 been expressed in stable transformants of baby hamster
kidney cells in 10 to 100 mg amounts of recombinant
protein. In addition pNUT plasmids have been
constructed containing the full length cDNA for oTF
and chimeric cDNAs for hTF/2N-oTF/2C and oTF/2N-hTF/2C.

15   Characteristics of the site-directed mutants
include: the D63S mutant does bind iron (contrary to
speculations in the literature) but much less avidly
than the wild type protein. For instance, this mutant
loses its bound iron on electrophoresis in PAGE gels
20 containing 8 M urea, whereas the wild type retains its
bound iron. The maximum in the visible spectrum lies
at 422 nm in contrast to that or the wild type at 470
nm. The G65R mutant binds iron less tightly than does
the wild type and has a visible maximum at 470 nm.
25 The K206Q mutant binds iron much more avidly than does
the wild type, as does its model, oTF/2C. Whereas the
red color of the wild type iron protein disappears
very rapidly in 0.5 M acetate buffer at pH 4.9,
containing 1 mM each of EDTA and NTA, the mutant loses
30 no color at all and requires pH 4 and 1 mM
deferoxamine to release its bound iron. The
apo-mutant appears to rebind iron more slowly than the
wild type protein. The visible maximum lies at 460 nm
for this mutant.

The full length recombinant hTF runs at the same
rate as the serum-derived protein on SDS-PAGE.

<u>Equivalents</u>

Those skilled in the art will recognize, or be
05 able to ascertain using no more than routine
experimentation, numerous equivalents to the specific
procedures described herein. Such equivalents are
considered to be within the scope of this invention
and are covered by the following claims.

CLAIMS

1.   A recombinant transferrin.

2.   Recombinant human serum transferrin.

3.   A recombinant half-molecule of transferrin
comprising at least the metal-binding domain of a
single lobe of transferrin.

4.   A transferrin half-molecule of claim 3, wherein
the single lobe is the amino terminal lobe of
human serum transferrin.

5.   A transferrin half-molecule of claim 3, wherein
the single lobe is the carboxy terminal lobe of
human serum transferrin.

6.   A mutant transferrin half-molecule comprising at
least the metal-binding domain of a single lobe
of transferrin, the mutant having a stronger
binding avidity for metal than the binding
avidity of natural transferrin

7.   A mutant transferrin half-molecule of claim 6,
which has a stronger binding avidity for iron
than natural transferrin.

8.   A mutant transferrin half-molecule of claim 7,
comprising at least the metal-binding domain of a
single lobe of transferrin wherein the lysine
residue at position 206 of natural transferrin is
replaced with glutamine.

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9. A eukaryotic expression vector, comprising a
    nucleic acid construct comprising nucleic acid
    encoding a transferrin or a transferrin
    half-molecule comprising at least the binding
05 domain of a single lobe of transferrin linked to
    appropriate genetic regulatory elements for
    expression in an eukaryotic cell.

10. A eukaryotic expression vector of claim 9,
    wherein the nucleic acid construct includes a
10 nucleic acid encoding transferrin signal sequence
    linked to the nucleic acid encoding the
    transferrin or transferrin half-molecule.

11. A eukaryotic expression vector of claim 10,
    wherein the single lobe is the amino terminal
15 lobe of human serum transferrin.

12. A eukaryotic expression vector of claim 10,
    wherein the single lobe is the carboxy terminal
    lobe of human serum transferrin.

13. A eukaryotic expression vector of claim 9,
20 wherein the transferrin half-molecule contains a
    glutamine residue at position 206 in place of the
    lysine residue of natural transferrin.

14. A eukaryotic cell line transfected with the
    vector of claim 9.

25 15. A baby hamster kidney cell line transfected with
    the vector of claim 9.

16. A method of metal chelation therapy, comprising administering to a patient a recombinant half-molecule of transferrin comprising at least the metal-binding domain of a single lobe of
05   transferrin in an amount sufficient to reduce circulating levels of the metal.

17. A method of claim 16, wherein the metal is iron.

18. A method of claim 17, wherein the transferrin half-molecule is a mutant which binds iron more
10   avidly than natural transferrin.

19. A method of claim 18, wherein the transferrin half-molecule contains a glutamine residue at position 206 in place of the lysine residue of natural transferrin.

15 20. A nonserum supplement for cell culture medium containing recombinant transferrin.
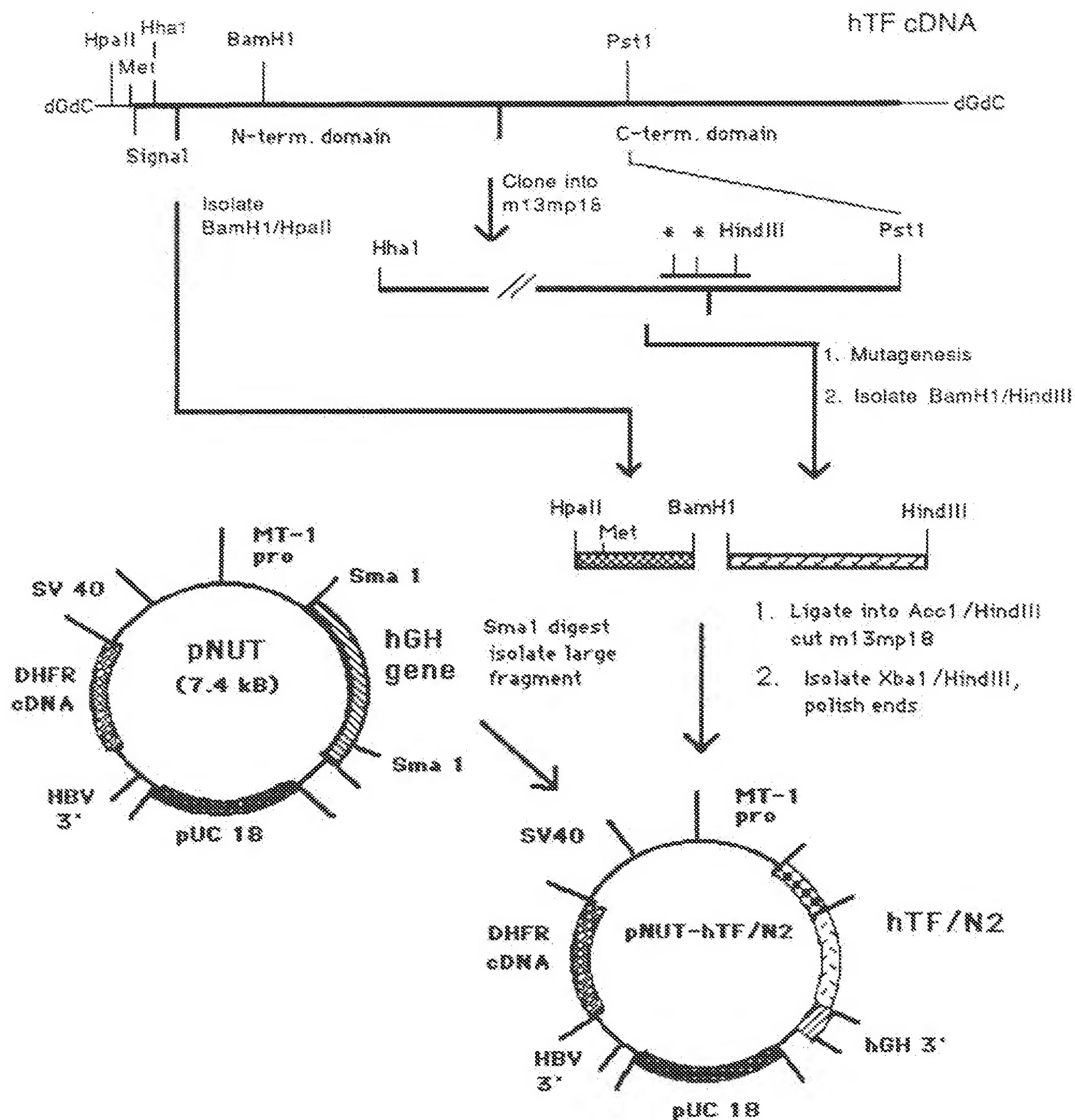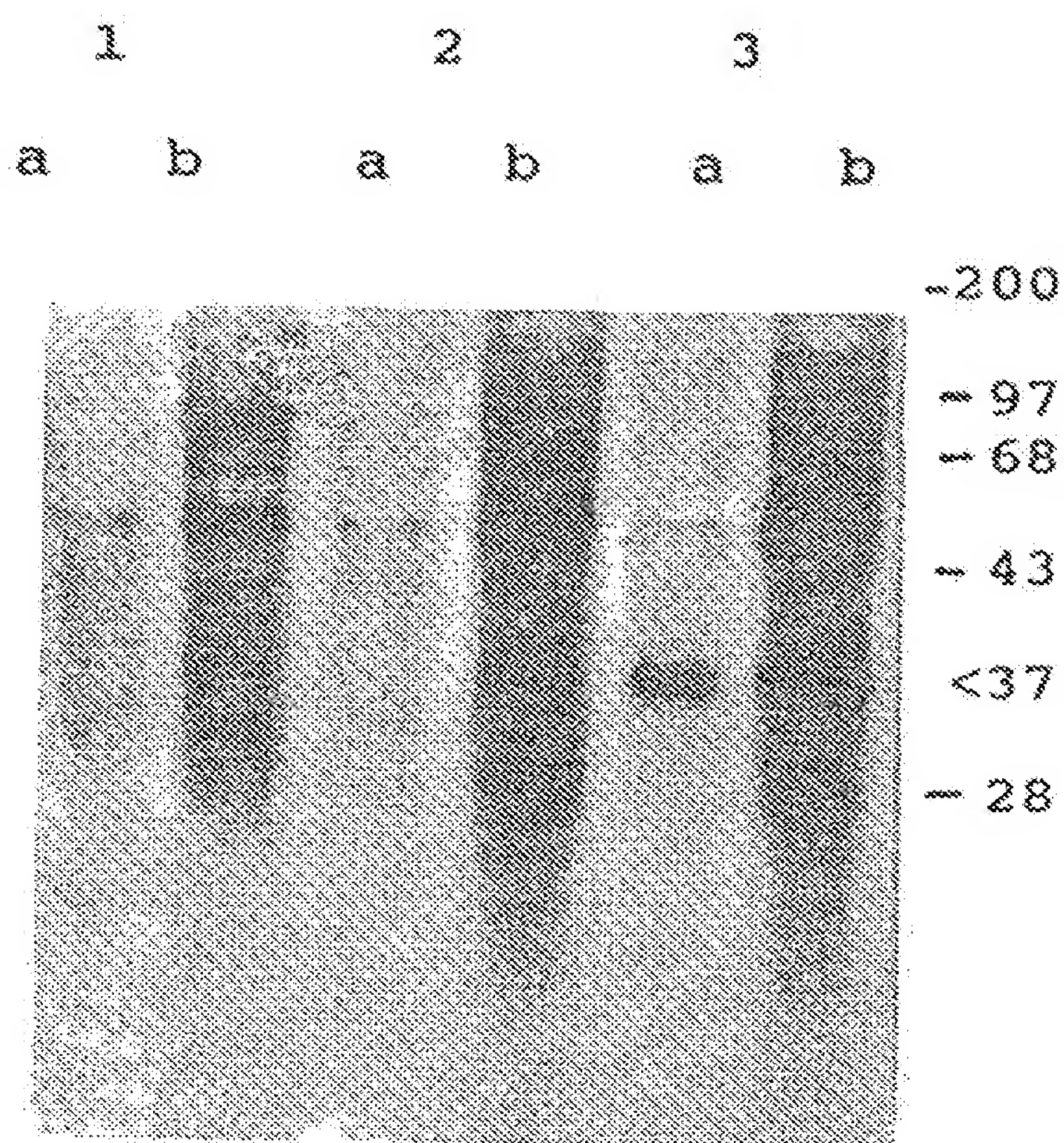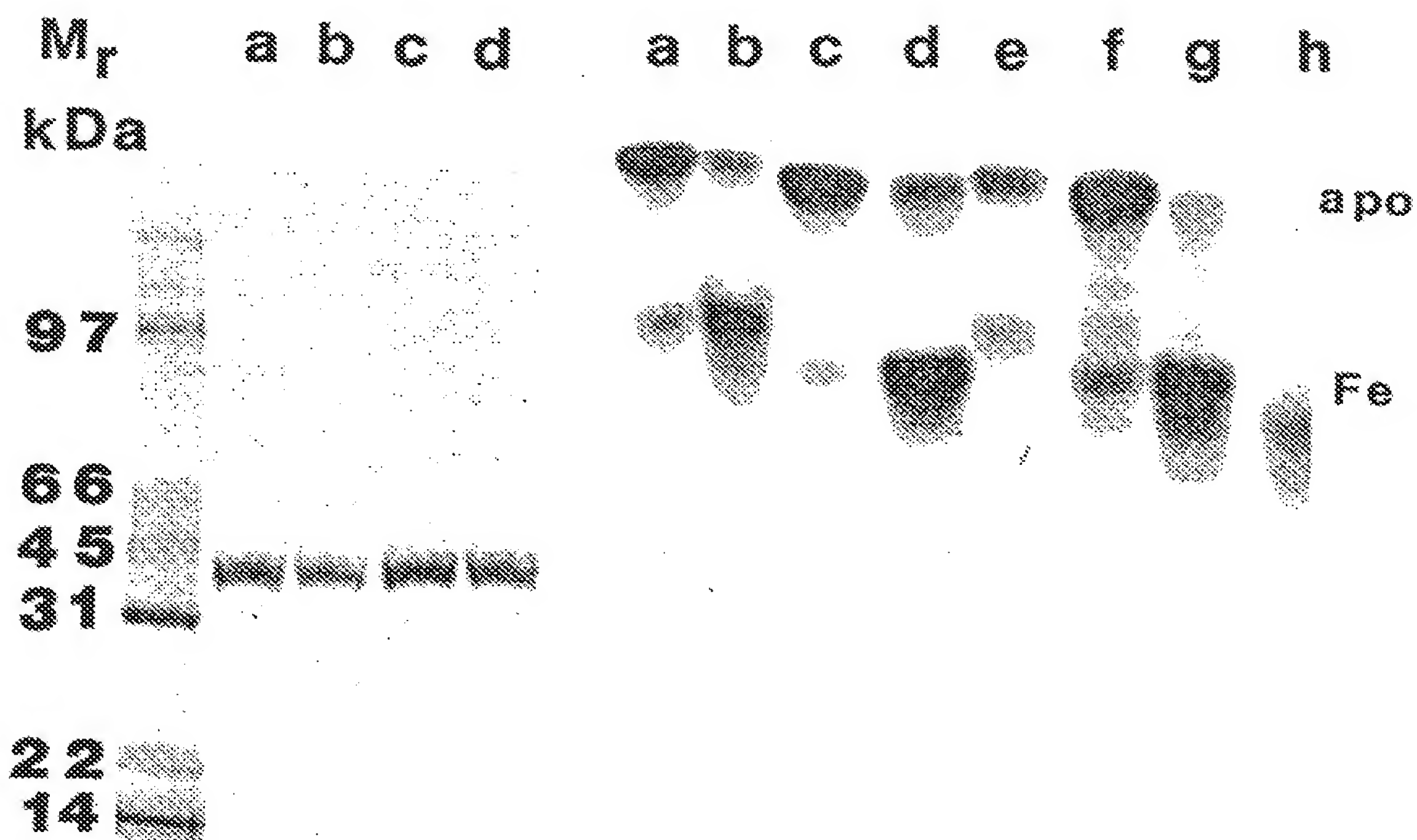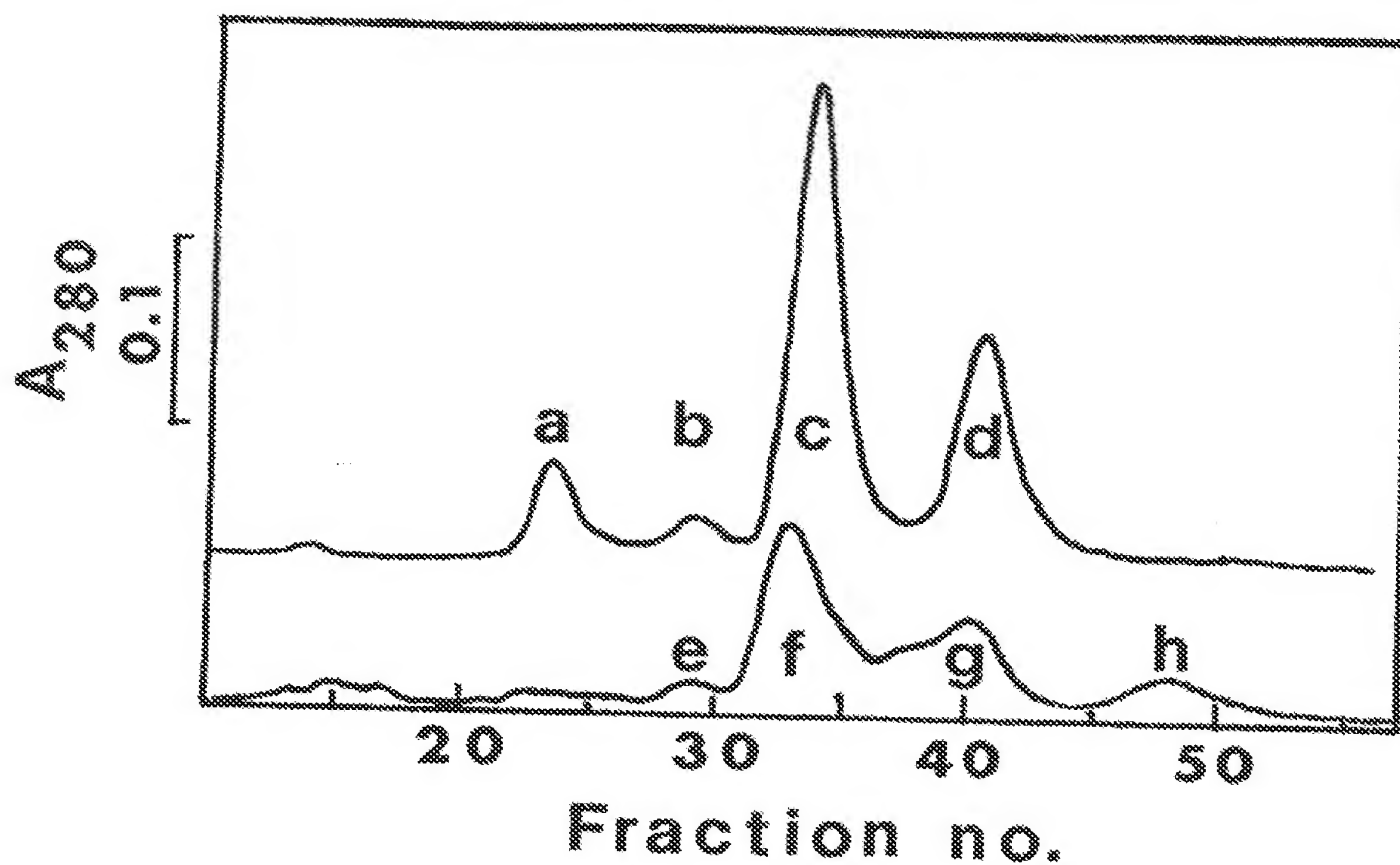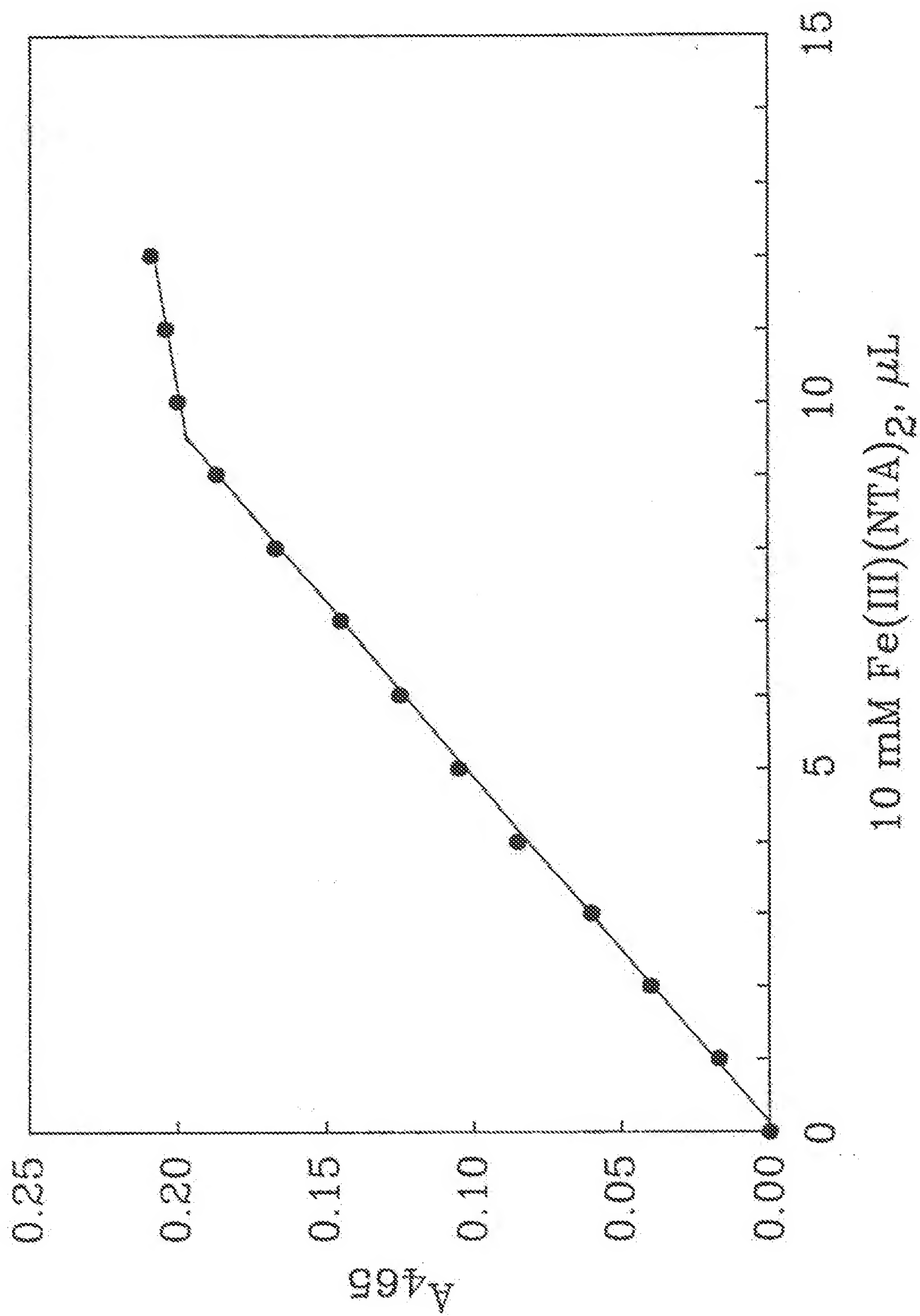
FIG. 1

FIG. 2

FIG. 3A

FIG. 3B   FIG. 3C

FIG. 4
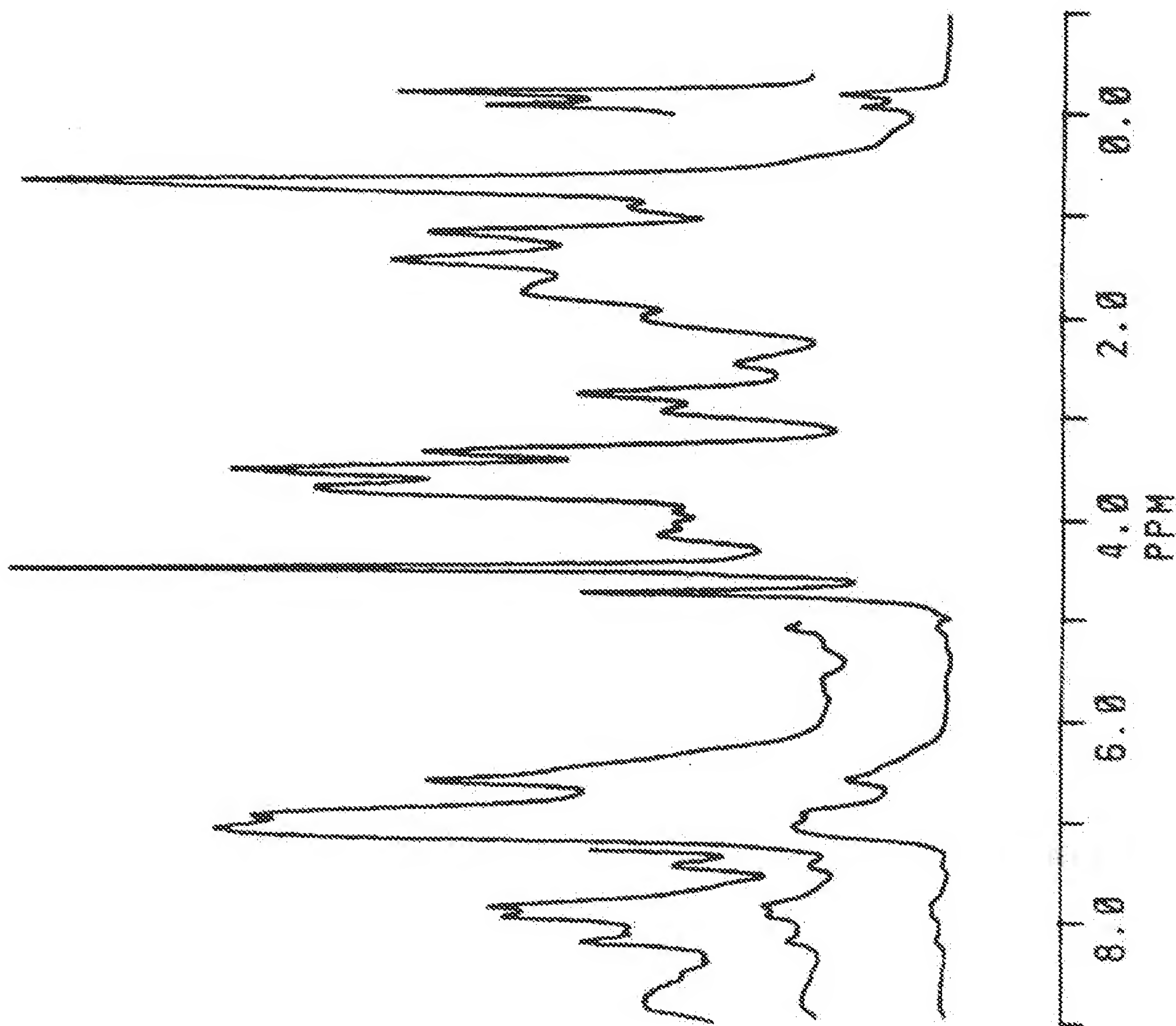
FIG. 5A
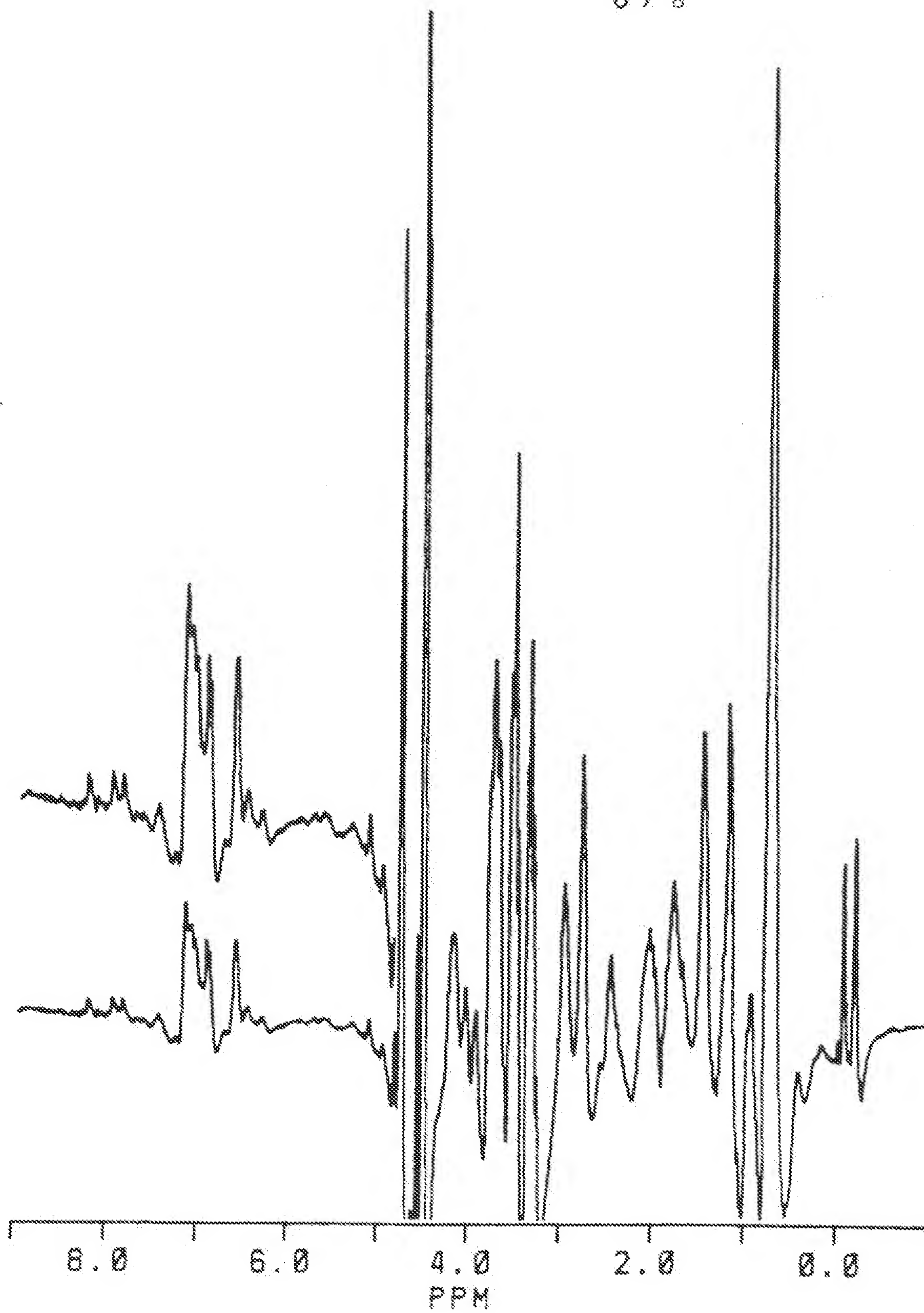
FIG. 5B
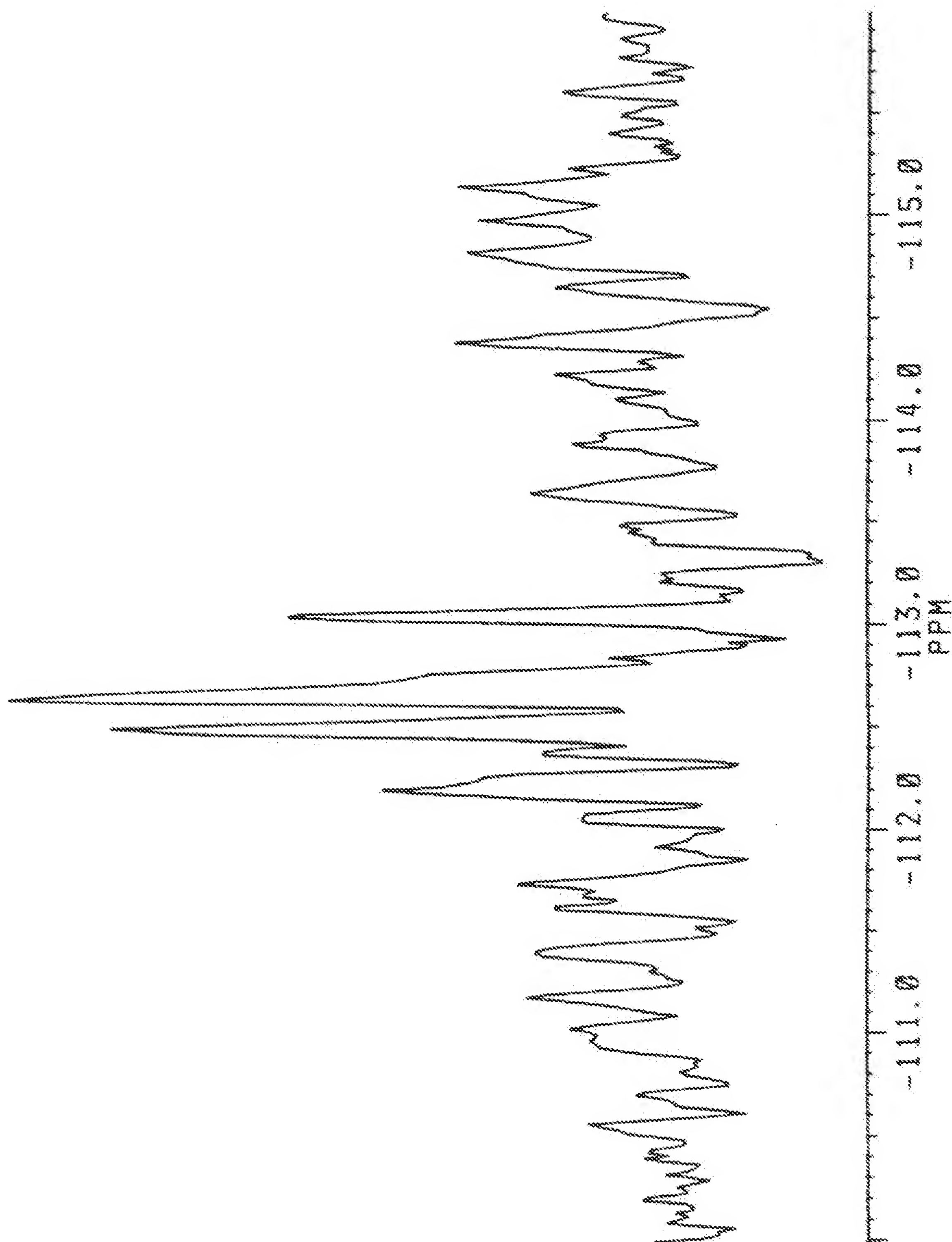
FIG. 6
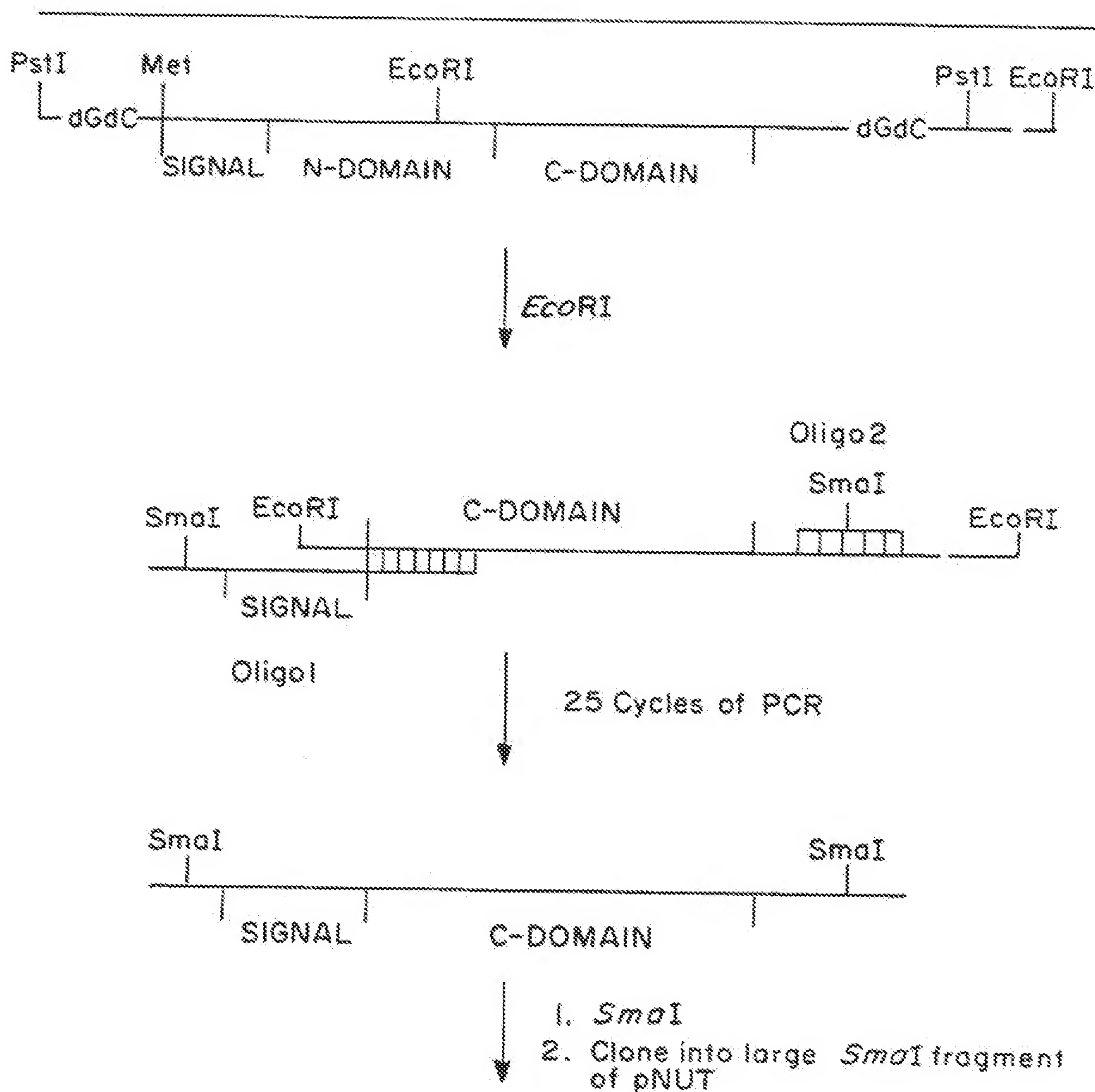
FIG. 7

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A 61K 37/02; C 07K 13/00
US CL : 514/6; 530/394

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	514/6; 530/394; 435/70.1; 935/9, 10, 70

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched[5]

APS, DIALOG:(MEDLINE, EMBASE, BIOTECH ABS) search terms: transferrin, sequence, production, purification, vector, human, metal chelation therapy, fusion protein, fusion peptide

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X	Biochemistry, Volume 29, issued 1990, W.D. Funk, et al, "Expression of the Amino-Terminal Half-Molecule of Human Serum Transferrin in Cultured Cells and Characterization of the Recombinant Protein", pages 1654-1660, see entire document.	1-4
X	Journal of Biological Chemistry, Volume 258, No. 6, issued 25 March 1983, R.T.A. MacGillivray et al, "The Primary Structure of Human Serum Transferrin", pages 3543-3553, see entire document.	1-2
X/Y	Journal of Biological Chemistry, Voulume 255, No.2, issued 25 January 1980, "Preparation and Characterization of an NH$_2$-terminal Fragment of Human Serum Transferrin Containing a Single Iron-binding Site", pages 708-713, see entire document.	3,4/5
Y,P	US, A, 5,026,651 (Bowman et al) 25 June 1991, see column 2, lines 45-52 and column 3, lines 41-52.	1, 2

* Special categories of cited documents:[15]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
14 APRIL 1992	12 MAY 1992

International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Gabriele E. Bugaisky

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	FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

    1-8 and 16-19 (Telephone Practice)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
    This ISA found multiple inventions as follows:

I.   Claims 1-8 and 16-19, drawn to transferrin and a method of therapy using
transferrin, classified in Class 514/6.
II.   Claims 9-15, drawn to a eukaryotic expression vector and a transformed cell,
classified in Class 435/320.
III.   Claim 20, drawn to a non-serum supplement for culture media, classified in
Class 435/240.3